

## An Efficient Method for Surface Sterilization and Sowing Fern Spores *in vitro*

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**ABSTRACT.**—Spores are commonly used to start *in vitro* culture of ferns. Numerous methods for spore surface sterilization and sowing have been developed, but spore loss and contamination are still problematic. To overcome these problems, an efficient method for sterilizing and sowing spores was established. Through this method, contamination and loss of spores is minimized, and can be sown in adjustable, even densities.

**KEY WORDS.**—surface sterilization, *in vitro* culture, spore sowing

Surface sterilization is the first step for aseptic culture of ferns from spores (Dyer, 1979). Since tissue culture is widely employed as a technique for fern propagation or scientific studies, spores are widely used as a starting material. In recent years, there have been numerous studies on *in vitro* culture of ferns from spores (Stone, 1958; Yoroï, 1972; Kiss and Kiss, 1998; Cox *et al.*, 2003). These researchers reported successful cultures, but admitted significant losses of spores during the sterilization process, aseptic sowing, or due to contamination (Warne *et al.*, 1986). Because of these problems, different methods of spore sterilization and sowing have been developed (Dyer, 1979). Many of these methods are still inefficient in terms of time and spore loss (Warne *et al.*, 1986).

Recently, we established an effective method for sterilization of spores with a filter funnel. The method is successful with spores of *Osmunda japonica* Thunb., *Aleuritopteris argentea* Gmel., *Adiantum flabellulatum* L., *Adiantum capillis-veneris* L. and *Cyrtomium fortunei* J. Smith (data not shown). We compared our method, here called the filter method, with two other widely used methods. With the packet method, spores were sterilized in filter bags/packets (Ford and Fay, 1999), while with the centrifugation method the spores were suspended in a sterilizing solution and then harvested in sterile distilled water by centrifugation (Fernández *et al.*, 1993). For these comparisons, we used spores of *Adiantum reniforme* var. *sinense* Y. X. Lin, a rare and endemic species in China.

### MATERIALS AND METHODS

**Plant materials.**—Sporophytes of *A. reniforme* var. *sinense* were introduced from Wanxian County along the Yangtze River in 2001 and cultivated in the

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greenhouse of Huazhong Agricultural University (Wuhan, China). Fertile fronds of sporophytes in the greenhouse were collected and wrapped in paper bags and dried at room temperature for one week to release spores. Then the spores were collected in centrifuge tubes and stored at 4°C until used. For this study, spores were stored for 10 months.

*Culture media.*—*Murashige* and *Skoog* (1962) medium (MS) with 1/4 strength of macronutrients were used for germination of spores. The medium was supplemented with 3% (w/v) of sucrose, solidified with 0.65% (w/v) agar and adjusted to pH 5.8 before autoclaving at 121°C and 1.1 kg cm<sup>-2</sup> for 20 min.

*Spore sterilization and sowing.*—The filter method was performed in a laminar flow hood. Three mg spores were suspended and wetted with 4% (v/v) Tween solution for 5 min in a 1.5 ml centrifuge tube. Suspended spores were collected through a filter funnel (made by fast filter paper), which was placed on a proper conical flask. The tube was washed three times with fresh water and the water was poured into the funnel to collect the residual spores of the tube. Seventy percent (v/v) alcohol (chemical purity) was added to immerge the spores along the funnel margin. Thirty seconds later 30 ml fresh sterile distilled water was continually added to rinse the spores.

After removing the alcohol, 4% (w/v) sodium hypochlorite (NaClO) or 0.1% (w/v) mercuric chloride (HgCl<sub>2</sub>) was added along the funnel margin. For different disinfectants, the sterilizing time was different: NaClO (5–6 min) and HgCl<sub>2</sub> (2–3min). At the end of the sterilizing time, fresh sterile distilled water was full filled into the funnel along the margin, regardless of whether the HgCl<sub>2</sub> or NaClO solution had completely drained off or not. The same operations were repeated twice when the dilute solution seeped half from the filter funnel. Then after the solution drained off, spores were thoroughly rinsed six times with sterile distilled water. After the sterile distilled water drained off, the spores were rinsed with 40 ml fresh sterile distilled water from the filter paper into a sterile container. Using a sterile pipette, the spore suspension was distributed onto culture plates (9 cm diameter Petri dishes, containing 20 ml culture medium), which were sealed with plastic film. The sterile distilled water and different solutions were transferred by transfer pipette with sterilized tips (1ml).

Ten plates were made for each treatment and the experiment was conducted three times. The waste of the HgCl<sub>2</sub> solution was collected and adjusted to pH 8–10. Enough Na<sub>2</sub>S and FeSO<sub>4</sub> were then added to react with the HgCl<sub>2</sub> and produce sediments. After sediments formed thoroughly, the sediments were collected and sent to the hazardous waste disposal department for detoxification and proper disposal.

All cultures were incubated in a controlled environment room that was maintained at 23±2°C under a light intensity of 25 μl m<sup>-2</sup> s<sup>-1</sup> with 16/8 photoperiod.

The spore density of one drop of spore suspension solution from a 1 ml transfer pipette in the filter method and centrifugation method were recorded and means with standard deviations (S.D.) were calculated. In the packet



method, spores were sown by directly wiping the spores on the medium, thus spore density was not scored. Contamination and spore germination were examined on the 40th day after spore sowing. For germination rates, at least 300 random spores per plate were scored.

For comparison to the other two methods, the published methodologies (Fernández *et al.*, 1993; Ford and Fay, 1999) were followed using 3 mg of spores, and are not described in this paper.

#### *Sterilization capacities of different sterilization methods:*

To test the sterilization capacities of the different methods, comparisons were made between different methods using different weights of spores (0.5g, 1.5g and 3g). The spores were administered into proper centrifuge tubes or packed in proper filter papers. All spores were sown in soil after sterilization.

### RESULTS

In the filter method, spores sterilized by NaClO were obviously bleached and it was difficult to judge whether the spores were rinsed completely from the filter paper or if spores drifted from the tubes when poured out of the disinfecting solutions and rinse water. Table 1 shows that there were no significant differences in spore density between the two disinfectants. However, the spore densities varied greatly between the different methods tested. From the centrifugation method, there were about 69–75 spores /drop, from the filter method, there were about 235–248 spores/drop, and the number of spores not sterilized and suspended in 40 ml water directly was 260–272 spores/drop (Table 1). In the packet method, sowing of spores was achieved by wiping the spores in a swirling motion over the surface of culture medium directly, so the spore density through this method was very high in the first plate and very low in the last plate. Nevertheless, the spores tended to clump together on the first plate. Thus, the spore densities changed greatly from plate to plate and across plates. Within a single plate, the highest density was more than 3000 spores  $\text{cm}^{-2}$ ; the lowest density was less than 10 spores  $\text{cm}^{-2}$ .

The spores from the filter method and packet method started to germinate 10 days after sowing, while the spores from the centrifugation method started to germinate 15 days after sowing. On the 40th day, the highest germination (62.6%; see Table 1) was obtained from the filter method with  $\text{HgCl}_2$ , which was followed by the filter method with NaClO (60.8%, Table 1).

The germination rate of the packet method varied greatly. For example, when the disinfectant was  $\text{HgCl}_2$  it ranged from 5.6% to 67%. At the highest density of about 3000  $\text{cm}^{-2}$ , germination rate was around 20%. The germination rate in the last plate was 5.6% as there were only 287 spores in the whole plate. Only plates from the packet method were contaminated (Table 1).

With increasing spore weight, different methods had different problems. For the packet method, when the spores were more than 0.5 g, removing air



TABLE 1. Effects of different sterilization methods and disinfectants on spore germination of *A. reniforme* var. *sinense*.

Disinfectant	Sterilization method	Sterilization time (min)	Spore number/drop	Contamination (%)	Germination (%)±S.D.	Spore color
NaClO	PM	5-6		36.7	40.1 ± 22.9	bleached
NaClO	FM	5-6	235 ± 7.8	0	60.8 ± 8.4	bleached
NaClO	CM	5-6	69 ± 4.6	0	27.4 ± 5.1	bleached
HgCl <sub>2</sub>	PM	2-3		23.3	39.9 ± 26.3	unchanged
HgCl <sub>2</sub>	FM	2-3	248 ± 5.7	0	62.6 ± 9.8	unchanged
HgCl <sub>2</sub>	CM	2-3	75 ± 5.5	0	23.2 ± 3.2	unchanged

Data of spore number/drop were taken after the sterilization; data of contamination and germination were taken after 40 days. PM: packet method; FM: filter method; CM: centrifuge method.

bubbles from the packet became very difficult, and some spores could not be wetted and sterilized. For the centrifugation method, when the spores were 0.5 g, 1.5–2 ml centrifuge tubes were proper; when the spores were 1.5–3 g, 1.5–2 ml centrifuge tubes were too small; 5–7 ml centrifuge tubes and a bigger centrifuge were needed. For the filter method, the spores, regardless of density, could be completely sterilized without any modification of the methodology.

#### DISCUSSION

When sterilizing spores via the packet method, the spores were kept in the packet during the sterilizing process. However, if the bubbles were not removed completely, the spores did not all come into contact with disinfectant and this caused an increased contamination rate. In addition, the sowing methodology caused the spores to be dispersed unevenly on the culture medium; some plates had spores that were clumped together and some plates had few spores that were spread very far apart. As a result of these discrepancies, the germination rates varied greatly, and confirmed the findings of Ashcroft and Sheffield (2000) that spore germination rate of ferns is inhibited at both high and low densities; proper spore density is important to fern culture.

When sterilizing spores via the centrifuge method, the spores tended to run off when pouring out the used disinfectant solution and used sterilized distilled water. Therefore, after the whole sterilization process, few spores remained, although the spores could be sown in an even density. The results of testing sterilization capacity with this method show that when the spore weight exceeded 1.5 g, larger centrifuge tubes and a larger centrifuge were needed.

When sterilizing spores via the filter method, the spores were kept in the filter funnel during the whole sterilizing process. Thus, spore loss was minimal. Besides this, the sowing spore density could be adjusted evenly through adjusting the volume of the sterilized water used to rinse off the spores from the filter paper.

Given these observations, we conclude that the filter method is an effective way to sterilize spores. It is not only simple and convenient, but can be used to



sterilize many spores at one time and it minimizes spore loss. It also allows spores to be sown in an even density.

The results of this study showed that both  $\text{HgCl}_2$  and  $\text{NaClO}$  were effective disinfectants. Since  $\text{NaClO}$  bleached the spores, it was difficult to judge whether the spores were rinsed off from the centrifuge tubes and filter papers thoroughly or not. However,  $\text{HgCl}_2$  is not only extremely toxic to spores but also to the environment. Thus, for normal *in vitro* culture, it is better to use  $\text{NaClO}$ .  $\text{HgCl}_2$  might be used in cases where the spores are difficult to sterilize or become too bleached. Because of the toxicity of  $\text{HgCl}_2$ , it should be handled very carefully, and the waste of  $\text{HgCl}_2$  should be detoxified or sent to a waste disposal department for detoxification.

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